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Effects of warming on the reproduction of the three-spined stickleback: behavioural responses and fitness consequences

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Summary

Climate change is changing the Baltic Sea. Eutrophication and rising temperatures are threatening its unique ecosystem. The three-spined stickleback (*Gasterosteus aculeatus*), a common model species helps us to understand the processes happening in the Baltic Sea. The main question is: are there changes in the parental behaviour and early development of the stickleback due to the warming environment?

The goal of my work was to observe the effects of elevated temperature on the reproductive success of sticklebacks: behavioural differences of parental care and the general condition of males, changes in the hatching success of the offspring and their survival probability throughout the first two weeks of their life.

Sticklebacks were caught and transported to the laboratory to reproduce. The behavioural responses of 32 males divided into two groups were observed during two reproductive cycles in two different temperatures. Videos of their parental behaviour were made daily. The weight of the males and eggs, plus the fry mortality and growth was measured.

In elevated temperature the males were forced to invest more energy in parental care, causing greater weight loss. Although hatching was more successful and faster at higher temperatures, the survival rate appeared to be lower. Early growth rate seems to be faster in the lower temperature. The changes in parental behaviour and trends in survival are likely to have consequences for the Baltic Sea three-spined stickleback population.

Language: English Key words: three-spined stickleback (*Gasterosteus aculeatus*), reproduction, parental behaviour, egg, fry, fitness, climate change, Baltic Sea, temperature rise

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1 Introduction

Carbon dioxide (CO_2) is a vital gas for life, but it is also a greenhouse gas which means it prevents the arriving energy of the sun from escaping the earth's atmosphere (Zielinski 2018). Since 1850 anthropogenic activities are constantly increasing the level of the CO_2, which plays an important role in regulating the atmospheric temperature by trapping the heat and manipulating the global climate affecting the life of all terrestrial and aquatic organisms (Laffoley & Baxter 2016, 22, Zielinski 2018). Warming, ocean acidification, growing ocean floor hypoxia, sea level rise, flooding, storms, more rain, salinity decrease, drought, and eutrophication are examples of the consequences of climate change (Laffoley & Baxter 2016, 34). In 2019, the European Commission called climate change an urgent challenge which should be acted upon promptly. Its impact is noticeable with increasing tendency in all continents (Laffoley & Baxter 2016, 100). Climate warming has triggered noticeable and measurable changes all around the world and the coastal waters of the Baltic Sea are no exception (ICES 2019, 11, Laffoley & Baxter 2016, 446). The Baltic Sea ecosystems are vulnerable due to the shallowness of its water body and the environment's unique natural properties (HELCOM 2013, 46). The average surface temperature from 1990 to 2008 rose by 1°C (ICES 2019, 11, HELCOM 2013, 20). The temperature together with the frequency and intensity of the heat waves, increased also in the entire Baltic, especially in the coastal areas (HELCOM, 2013, 47). The milder winters and warmer summers alter the hydrography, biogeochemistry, and physical properties of the Baltic Sea (ICES 2019, 11, 41). As a consequence the Baltic Sea fails to reach the pre- 1960s good environmental state despite the environmental measures (HELCOM, 2013, 52).

Rapid changes combined with anthropogenic disturbance can decrease the survival of many species and modify the behaviour of the animals (Candolin et al. 2014). The three-spined stickleback *(Gasterosteus aculeatus)* has been shown to be affected by the changed temperature (Candolin et al. 2006). Climate change, pollution, overfishing of top predators (cod *Gadus morhua*, pikeperch *Sander lucioperca*, and perch *Perca fluviatilis*) and habitat destruction affect dynamics and behaviour of many other species too (Candolin & Voigt 2020). It is known that the breeding habitats and the mating rituals as well as the parental behaviour of the stickleback are changing (Candolin & Voigt 2020); yet little is known about how much and in what direction (Stein & Bell 2012), despite the fact that the species is probably one of the winners of the changed circumstances.

The three-spined stickleback is an excellent and frequently used model species of the littoral zone in biological researches. Therefore its reaction to the rising temperature could give us valuable information how the environment and the ecosystem will be affected. However, the knowledge about the impact of environmental changes on the stickleback is insufficient (Stein & Bell 2012). Improvements are needed in order to get further understanding on the global effects of the climate warming and extreme temperatures.

2 Background and research questions

Studies show that the behaviour of the male stickleback changes due to environmental stimuli, for example presence of a predator, regardless of the genetic background (Huntingford 1976). Changes in the environment can adjust the direction of the evolution of the sticklebacks through manipulating sexual selection and pushing altered pressure on different aspects of the life stages (Candolin et al. 2006).

The main hypothesis of my thesis is that increased temperature affects the reproduction and mating behaviour of the three-spined stickleback. It is known that behavioural changes are among the first reactions after altered conditions in many species (Wong & Candolin 2015). Together with the courting performance differences, change in parenting behaviour might affect the survival and growth success of offspring. If the changes in mating and parental behaviour disturb or redound the reproductive cycle and alter the development of the juveniles (Wong & Candolin 2015), the population size is probably influenced (Candolin & Voigt 2020).

The offspring hatching rate is correlated with the time the males spend taking care of the nest (Wootton 1984). If energy (cost) invested into reproduction exceeds the benefit due to a change in the environment, altered parental behaviour may have a negative impact on the species since there is a positive correlation between male mortality and reproductive effort (Wootton 1984, 235). Glippa et al. (2017) have shown that higher temperatures result faster growth of juveniles, but the growth rate seems the decrease when temperature raises over 19°C (Wootton 1984). If the fecundity rate increases as Candolin and Voigt (2020) have observed and the growth rate of the offspring increases, it is likely to have positive consequences on the size of the stickleback population. The males are fully responsible for taking care of the offspring, possibly having the greatest impact on the new generation and consequently on the population (Stein & Bell 2012).

To test the hypothesis, another student and I conducted a 47 days long study, which is based on 10 min. behavioural videos recorded for later analysis and direct size and weight measurements. The questions of my thesis are:

- Does fanning of *Gasterosteus aculeatus* differ in elevated temperature, or is fanning due to individual differences? Does this indicate greater weight loss for the males?

- How do the observed differences affect offspring survival and development?

Based on these research questions the following null hypotheses were formulated: $H0_1$ (Null hypothesis 1) the temperature has no significant effect on stickleback parenting behaviour and fitness of the males and offspring, $H0_2$ (Null hypothesis 2), there will not be observable differences between the two cycles, $H0_3$, (Null hypothesis 3) there will be differences within the same group due to exchanging males.

Unlike other water parameters, changes in temperature and oxygen (O_2) can have a profound effect on the stickleback' early development since the developing embryos' most important demand is the oxygen which needs to be supplied by the male itself (Glippa et al. 2017). The expectation is that elevated temperatures will speed up the metabolism of adults and the larvae (Van Iersel 1953, 78). The dissolved oxygen (D.O. in mg/L) level is dependent on the water' (H_2O) temperature, pressure and salinity (Fondriest Environmental 2013). The oxygen is less soluble in warmer water which in practise means that the dissolved oxygen is 9.993 mg/L at 100% air saturation and 14°C, and 9.005 mg/L at 19°C in Baltic Sea conditions (salinity 4-6) (Fondriest Environmental 2013). The variability in the oxygen level might motivate males to apply more parenting effort. Males are cleaning and fanning the eggs in order to support the hatching success, as the eggs and the juveniles are sensitive to changes in oxygen and temperature among other factors (Van Iersel 1953, 77, Glippa et al. 2017). This potentially decrease male survival and worsen their condition for the next breeding round. The experiment might help to answer how the male sticklebacks respond to temperature fluctuations during the reproductive period. The whole study covers different reproductive aspect of the species, but my thesis focuses mainly on the parental behaviour and offspring survival.

The species has been studied since 1994 at Tvärminne Zoological Station by Candolin and collaborators. The significance of males' parental behaviour has been studied several times (Stein and Bell 2012). Bell et al. (2009) reported that the variability in behaviour and individual responses often can be based on genetic background instead of the effects on the

environment. Bell & Stamps (2004) found that some behavioural patterns of the stickleback changed due to the environment effects and some did not. Olsson et al. (2019) showed that there is a lack of knowledge on stickleback population biomass and suggested that the species must be monitored and researched more.

The stickleback stock in the Baltic Sea has increased a lot in some areas throughout the last few decades (Olsson et al. 2019); one likely reason is the increased reproduction rate, and offspring production (Candolin et al. 2014), combined with faster larval growth rate at warmer temperatures (Glippa et al. 2017). The breeding habitats in the Baltic Sea are continuously changing due to changes in the algae community caused by eutrophication, which might affect the breeding success of the stickleback (Candolin et al. 2006). Candolin et al. (2014) studied the direction of sexual selection of the stickleback in an altered environment which turned out to favour smaller males. Candolin et al. (2006) observed that excess growth in macro algae community is disadvantageous for males, as the energy requirement for building the nest was significantly higher. On the other hand, in another study Candolin et al. (2005) showed that the excess algae growth could help the males' parental success by helping to avoid predators and competition. Turbid water, which can be a result of a phytoplankton bloom, affected females' ability to choose the right mate. As a result, it affects the survival rate of the offspring, weaker males with weaker parental instincts are more likely to cannibalise the eggs (Wong & Candolin, 2015).

3 The subject animal

The subject of the research is the three-spined stickleback *(Gasterosteus aculeatus,* Linnaeus, 1758*),* which is a common and abundant species in the northern hemisphere, also in the Baltic Sea but not in central Asia. The stickleback has many subspecies adapted to different environments. The species can undergo rapid evolutionary changes leading to high variation (Olsson et al. 2019). Its variability and adaptation to numerous kinds of different habitats makes it a perfect model animal for experiments (Wootton 1984, 238). The importance of the stickleback lies within its wide tolerance. It is an excellent subject due to its complex behaviour and responses to ecologically and environmentally relevant changes (Stein & Bell 2012). Its interactions with other species in the community such as predators and parasites can give valuable information regarding the environment as whole (Wootton 1984, 238). The species is a generalist carnivore preferring aquatic invertebrates (Hagerty 2015). Regardless of, the species' wide distribution ranges and it's ranking by the IUCN as least concern, the isolated freshwater subspecies' status requires further investigation. The

population of these isolated subspecies is not as widespread and could be limited to fewer individuals.

3.1 Biology

The three-spined stickleback is a tiny fish with a life expectancy of ca. 3-4 years. Some populations can become sexually mature within 2 years but the species usually complete only one reproductive season (Candolin 2000). The reproduction period begins in early spring. In the Baltic Sea it occurs approximately during the first or second week of May and continues until late July (Candolin et al. 2006, Candolin & Voigt 2020). During the reproduction period the sticklebacks migrate back from the deeper water bodies towards the coast (Van Iersel 1953, 4, Wootton 1984), where the males start to occupy territories and build their nests from algae (Stein & Bell 2012). The poor-quality males may never build their nests, not even in isolated tanks where competition is absent (Wootton 1984), the reason is not entirely known. The males are polygamous and invite as many females as they can (Wootton 1984, 153). Their unique courtship behaviour is specific to the species. After laying the eggs the female must leave, otherwise the aggressive male will attack and kill her (Wootton 1976, Stein & Bell 2012).

The 60-100 fertilised eggs in each clutch hatch in 4-9 days depending on the temperature (Stein & Bell 2012). The nest is protected, taken care of and oxygenated by the male (Huntingford 1976, Stein & Bell 2012). Without the male's fanning, the eggs would suffocate very soon (Van Iersel 1953, 25). The reproductive strategy is very costly for the male (Wootton 1976). He provides all the necessity required for the eggs to survive and therefore the hatching success is dependent on the male's parenting ability (Stein & Bell 2012). The male does not feed during this period as he spends all his time to guard the territory from other males, avoiding predators and taking care of the eggs (Wootton 1984, 236, Stein & Bell 2012).

3.2 Morphology

The three-spined stickleback is a small, few centimetre long torpedo-shaped fish (Figure 1), similarly to the other members of the *Gasterosteus* genus (Wootton 1976). Its size is 2.5-8.5 cm, (max. 11 cm) in the Baltic Sea (Olsson et al. 2019). Depending on the subspecies, 2-4 spines are found on the back, from which the species originally got its name. Freshwater populations differ in morphology from the marine ones depending on the habitat (Hagerty 2015).

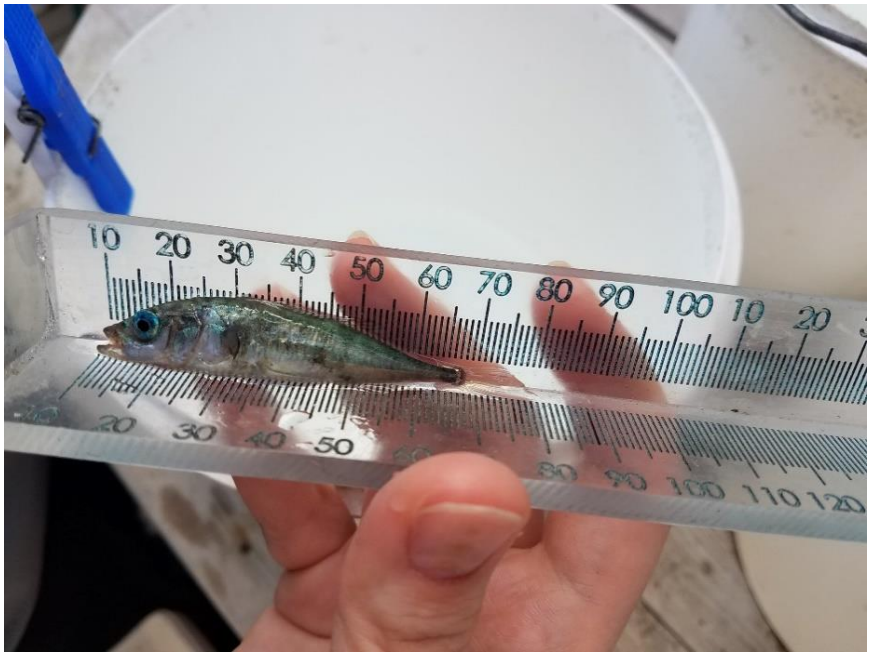

Figure 1. Stickleback male in breeding condition from the Baltic Sea population (Teija Isotalo, 2019)

3.3 Occurrence and environmental preferences

The geographic distribution covers North America, Europe all the way to Iceland and Greenland, Western Atlantic Ocean and the Eastern Pacific coast. The stickleback was introduced to continental waters in both North America and a large part of Europe. The stickleback can survive in various environments, and occurs in brackish, marine and fresh water from small streams to huge lakes at any altitude usually in shallow, but at some area even offshore water areas, covered with vegetation (Hagerty 2015). Some populations are anadromous, which means they migrate between marine and freshwater bodies, whereas others are adapted to the freshwater life entirely (Wootton 1976).

3.3.1 Local environment

The three-spined stickleback has the ability to tolerate a wide range of salinity (Wootton 1976) so it managed to adapt to the brackish water of the Baltic Sea (Figure 2). Here the stickleback lives mainly in the coastal waters. In areas with high precipitation the littoral zone's salinity and temperature are generally fluctuating, but in the Baltic Sea there is also wide seasonal difference, which makes its environment less stable. The low number of species, which is around 230 fish species, from mixed origin partly marine and partly freshwater, makes the ecosystem fragile and less resilient to changes (Olsson et al. 2019, ICES 2019, 14). The low biodiversity caused by strong salinity gradients, small changes can have larger effects on the ecosystem as whole. (Olsson et al. 2019). The 420 000 km^2 enclosed Baltic Sea with many islands is sensitive to eutrophication because of its shallowness (average 60 m) (ICES 2019, 1, (Laffoley & Baxter 2016, 40). Moreover, the species has to face daily, seasonal and annual temperature fluctuations and a long ice cover period (Furman et al. 2014, ICES 2019, 3). The northernmost stickleback population density can be found in the Baltic Proper and the Bothnian Sea; however, its exact distribution is not entirely known (Olsson et al. 2019).

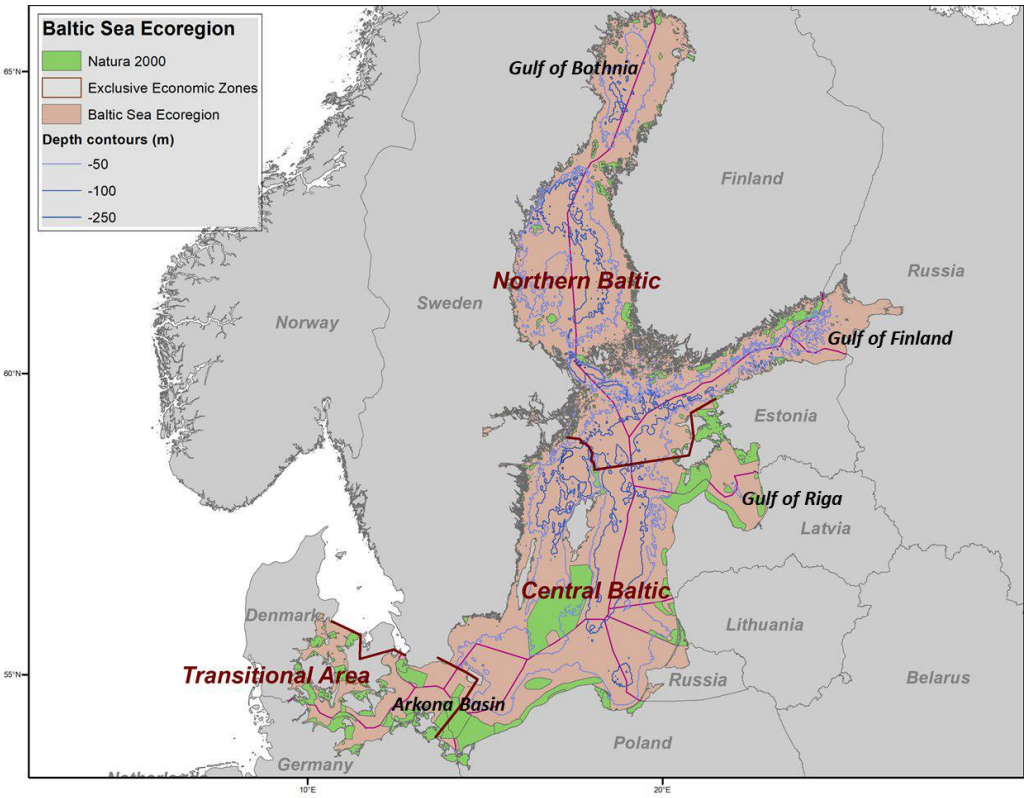

Figure 2. Baltic Sea ecoregion with water depth, stickleback habitat (ICES 2019).

4 Methods and procedures

The 47 days long experiment started 6 May, 2019 at Tvärminne Zoological Station located near Hanko, south-western Finland under the supervision of Ulrika Candolin, University of Helsinki. Approximately 80-100 healthy sticklebacks of which 40 females and 40 males in reproductive condition were needed. The number had to be higher than the actual number of individuals participating in the study, as not all individuals were healthy or willing to reproduce. The stickleback is known to have many frequently occurring diseases. Many fish were discarded because of their health condition. Prior to the research the Animal Experiment Board in Finland (ELLA) accepted the research plan according to the expectations of the Finnish legislation of animal welfare and the study was conducted under the control of the university veterinarians.

4.1 Pre incubation

As the first step, Plexiglas traps ($20 \times 20 \times 40$ cm) were set up in the shallow water, approximately 60-80 cm deep in Tvärminne, at Vindskär lagoon, to capture stickleback individuals from the local population. The catching procedure started early when the fish arrived at the coast before the start of their breeding season. The timing is important because they should not have any breeding experience before the test. Their previous experience in natural conditions could influence the outcomes. To speed up the procedure, a hand trawl was used alongside the traps in the neighbouring bays of Tvärminne. The area is a general breeding site for sticklebacks (Candolin & Voigt 2020) that provide all the necessary conditions for successful breeding, such as algae cover and hard, rocky and sandy bottom.

The fish were placed into temporary tanks divided according to sexes if the sex could be determined. The water temperature in the tanks were similar to the natural conditions, no heating or lighting was used. The determination of the sexes was sometimes complicated. Generally, the males have blue eyes with red bellies (Wootton 1984, 119), the females are grey coloured and have bigger bellies. The individuals with large belly volume, which otherwise may well be a female carrying eggs, may be infected by a parasitic tapeworm *(Schistocephalus solidus).* The fish were kept in the holding tanks until they reached the reproductive state and they were fed with frozen chironomid larvae (*Chironomus* sp.).

4.2 Preparation of the rooms and the tanks

For the experiment, the station provided two climate rooms, where it was possible to adjust the temperature individually. When the number of fish was satisfactory the setting up of the incubation tanks started. Non transparent white walled aquaria (Figure 3) with the volume of 10 litres each were used. Each was occupied by a randomly selected male with its own number, between 1 and 32, to be definitely able to distinguish them from each other. The rooms and tanks needed to be ready before the fish arrived, as it took several days to gather all material and supplies. The fish were separated into two groups with different temperature; one had 14°C water temperature which represents the normal conditions for the Scandinavian populations (Candolin et al. 2006) and one with 19°C water temperature which indicates the fastest growth rate for stickleback (Wootton 1984, 87), to imitate the effects of warming and demonstrate the changed conditions caused by climate change. The water flow needed to be moderate, or else it would negatively affect the tank temperature. Even with slow, and only just tipping water, both climate rooms had to have +2°C higher air temperature than the target temperature of the aquaria. The number of replicates per treatment were 16, which later in the second cycle were divided into two subgroups. If a male died or was discarded from the test, he was replaced by a new male with a new number (33-).

Figure 3. A tank with nest prepared for a male.

To guarantee that any detected differences are due to alterations of temperature and not from the individuals themselves, 50% of the fish were moved to the other temperature group after the first cycle as Stein and Bell (2012) did.

4.2.1 Water supply

The salinity of the water was similar to the natural environment (salinity 6). Apart from temperature, water parameters were not measured because the study did not focus on that and the species is not particularly sensitive to the chemical composition (pH, N and CO_2) of water (Glippa et al. 2017). The tanks had constant water flow and aeration; the water used for filling the tanks was pumped nonstop from the sea. The running water supply was necessary to keep the water clean and to have the same properties of for example O_2 and Co_2 as the water in the sea. The intensity of the fanning depends on the oxygen level of the water (Van Iersel 1953, 93), therefore the results would be influenced, if the oxygen level would differ greatly from normal. The extra water was drained with tubes from the tanks. The temperature was measured daily in each tank.

4.2.2 Lighting

The tanks were illuminated by Aqua Medic, Aquarius 120 plant LED (128W) lamps, exclusively manufactured for aquaria. The lamps were set up approximately 20 cm above the tanks (Figure 4). The light period at the beginning was 18h of light at 30% of the capacity of the lamp, which equals around 2700 Lumen. This included 1h sunrise and 1h sunset dimming between 0-30% capacity of the lamp and 6h of darkness. The lamps were programmed with Aqua Medic, Aquarius 6-channel controllers. Full spectrum light of the lamps meets the natural conditions applying to the region of Tvärminne, Finland in May-June. The illumination time was continuously adjusted every second week with 30min. because the days are prolonging during the season. Too strong light must be avoided due to the stress caused in the fish.

Figure 4. The fish tanks equipped with lighting.

4.3 Data collection

After the temperature had settled and all tanks were at approximately in correct state, the nesting material was placed into the middle of the tanks. The nesting materials (Figure 5) in each tank included; a dish filled with sand, a plastic plant and fresh filamentous green algae (*Cladophora glomerata*). The nest was expected to be built here. When the number of the caught stickleback was high enough, the males were introduced into the tanks. Their size and weight were measured. To motivate them to build a nest faster, females were introduced to them about twice a day for 15 min., in tiny perforated containers, so the males could sense the female odour and see them. Subsequently, the males got into reproductive condition faster which motivated them to develop deeper colour and to build their nest if they had not done that yet. Also, it was possible to detect if the male was suited for the study.

Figure 5. Tray with sand as nesting material and a plastic plant.

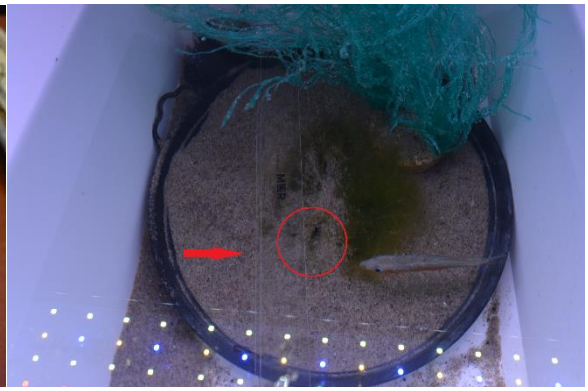

Figure 6. The ready nest with clear and visible entrance.

The first male started to build the nest 14 May and the last one the 20 May. The males which did not build a nest or were not interested in the females were replaced. The nest was considered ready when the entrance (Figure 6) where the couple creeps in, was clearly observable (Van Iersel 1953, 17).

4.4 Breeding and recording

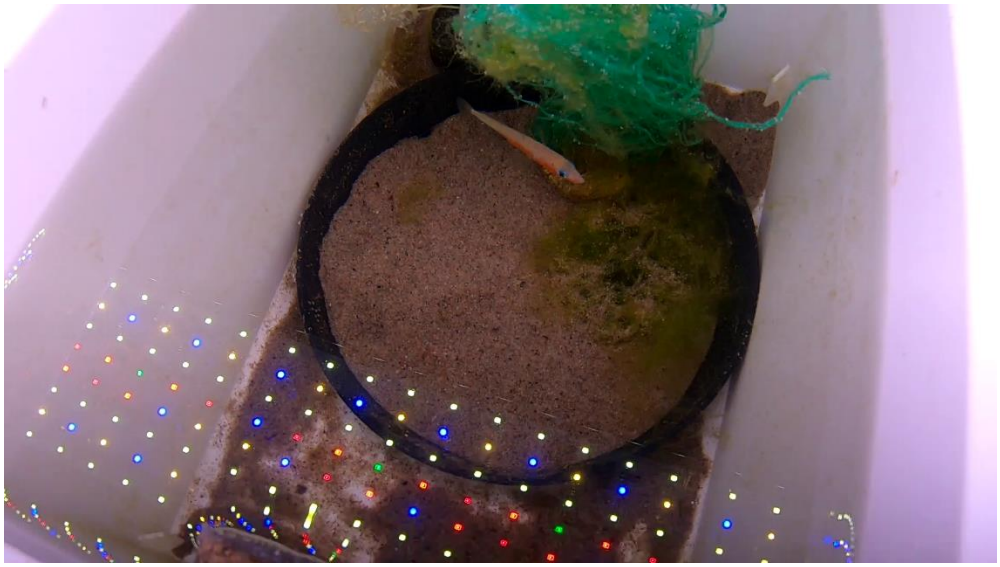

Figure 7. Male 31 in red coloration is guarding its nest.

When the males seemed ready, the real test began. The males were provided with one female at a time. At this point size and weight of the male and the eggs clutch were documented. The courtship behaviour was recorded with three different females and with the last female they were able to breed. If the female did not lay eggs, she was replaced by a new randomly chosen female. The females were placed in separate containers between the courtship experiments. Each courtship video (Figure 8) was 10 min. long and the males were given a 30 min. break after each session. When the 10 min. had passed, the female was placed next to the following male. When the male had his third round, the female was released into the tank and they were allowed to spawn. When the female had laid her eggs she was removed from the tank, and her body weight and size were recorded. They were returned to their original tanks and saved for the next breeding cycle. If the female did not lay the eggs within 2 hours or released the eggs before, the female was replaced by a new female. In these cases, a short time later, the process needed to be repeated.

Figure 8. Recording behaviours with the GoPro (Teija Isotalo, 2019).

Each male had only one female as a mating partner so the nest contained only one clutch of eggs. All males had to complete both reproductive cycles and have two egg clutches, one in each cycle. Data from males that died during the procedure were not used.

4.4.1 Eggs

After the spawning the males started to take care of the eggs. Two hours later when the eggs had hardened enough, they were gently removed from the nest and weighted, the egg number was not counted, after they were transferred back to the nest. The egg diameter was measured under the microscope by selecting five eggs randomly from each clutch, which can give some observable differences among the different temperature groups. Then the eggs were left with the father who accepted them back immediately. The eggs development is slower in the colder temperature and speeds up as the water warms (Van Iersel 1953, 59). Van Iersel (1953, 78) also states that the hatching time in 18.8°C happens at the 6.6th day, so 6 days in 19°C, and 8 days in the 14°C room seemed reasonable. Before hatching the eggs were measured once again and from the weight difference the mortality rate was estimated.

4.4.2 Fry

The removed eggs were placed in small, one litre tanks with aeration. Seven days after they hatched they were photographed (Figure 9), using n=5 individuals per time, and measured (approximately the standard length). Their number was reduced to 20, if there were more eggs in each tank. The rest of the juveniles was released back to the sea. The measurement was repeated one week later, the larve were counted and measured to observe their growth rate. They were fed with smashed brine shrimp *(Artemia salina)* larvae, one third of the water was changed every second day. The extra fries were released back to the sea near the station. The juveniles that were alive after 2 weeks were transported to Viikki, University of Helsinki, for further studies.

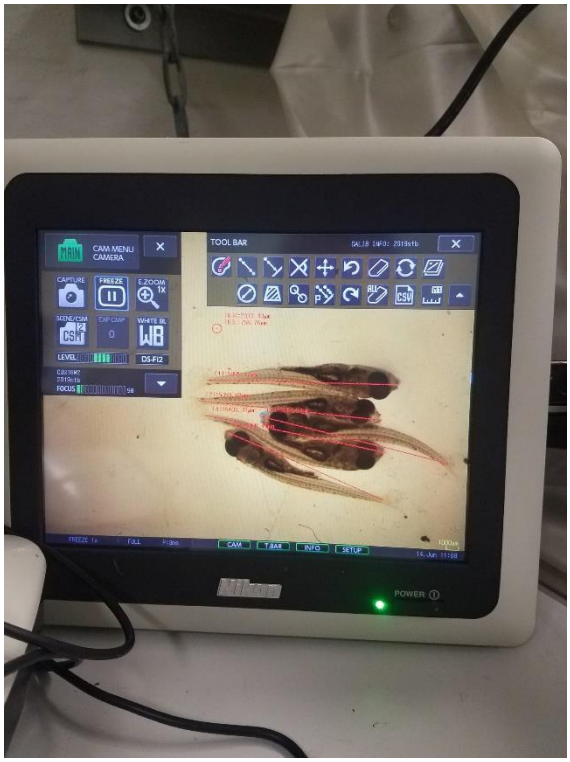

Figure 9. Measuring the length of the fry using microscope (Teija Isotalo, 2019).

4.4.3 Incubation and end of cycle

Throughout the incubation period the males did not get any food, only between the breeding cycles, as they would not feed in the natural environment either. The weight of the males was measured before and after the breeding season to estimate their weight loss. The parental behaviour was recorded every day for 10 min. After the first cycle, 50% of the males (8 individuals per group) were randomly switched to the other group to see if the temperature indicated the differences or only variance between the individuals? All males were given

new nesting material. Minor cleaning work was done daily by getting rid of the algae from all tanks. After the first breeding cycle was completed the tanks were cleaned, the water was partially exchanged and new, fresh nesting material was added. The procedure is unlikely to affect the results.

4.5 Data analysis and sample characteristics

I analysed the parental videos. The results are presented below with mean, standard deviations (S.D.) and standard error (S.E.) if needed. Each male was filmed every day, which means 4-8 10 min. videos (± few seconds) per cycle, depending of the temperature group. The length of the videos varied but there were no major differences. The videos were analysed using BORIS (Behavioural Observation Research Interactive Software). The program allows to measure the time and portion of a certain behaviour in relation of the video's interval. With the program four key behaviours were documented: Duration and frequency of fanning, no parental care and nose in the nest. The results from BORIS were exported as an Excel file. The videos did not track every moment or all aspects of parental care, so conclusions drawn are based on the assumption that the video sampling is representative for the whole study period, meaning that the events occurred similarly for the rest of the time.

To test my hypotheses that different temperatures produce different results, statistical methods were used; Friedman's one way ANOVA (analysis of variance for repeated measures $p<0.05$) Changes in the parenting phase were expected due to the temperature differences. The *t* test (two samples and pairwise *t* tests where $p<0.05$) was used to analyse the weight change of the males, the weight changes of the egg clutches and the growth rate and mortality of the fry, depending if the data were independent or not. All above were tested with IBM SPSS® 22 (Statistical Product and Service Solutions). The Friedman's ANOVA test does not require the data to be normally distributed (non-parametric test), therefore those results not shown below.

5 Results

As expected the males arrived to the coast earlier than the females, so the collecting went smoothly, and the experiment could start in time. The first spawning occurred 17 May in the 19°C climate room. Slightly later spawning occurred also in the 14°C climate room 20 May. Every male had completed his second spawning by 13 June. The males standard length (from the front of the head to the end of the tail without fins) was 63.4 ± 3.75mm (mean ± S.D., n=36), and the start weight was 2.47 ± 0.52g (mean ± S.D.). Apart from the differences between the individuals, it was in the beginning observable that the progress was slower in colder temperature. When the males built the nest within a day or a few hours in the 19 °C climate room, the 14°C males needed up to a week. If the female was interested, the spawning occurred quickly, usually within 15min. If the spawning did not happen during this time, the spawning did often not occur at all.

During our experiment 5 males died of which one male was in the 19°C treatment and four males in the 14°C climate room. Out of the five males which died, four were replaced and one managed to be present far enough in the process to give an acceptable result. No male was discarded due to the lack of nest building behaviour. The final sample size in both cycles was n=16 in the 19°C, and n=15 in the 14°C group.

Three males in 19°C and five males in 14°C cannibalised their eggs. Following the advice of my supervisor Ulrika Candolin, the data from these males were used as cannibalism is a natural behaviour. In these cases, the information from the egg weight loss and fry development were lost and the number of survivors is zero. Problems occurred with early hatching, when the young started to hatch earlier than expected. Even so, the young in the nest were in danger of being consumed by the male, the weight of these offspring could not be measured. The juveniles were immediately removed from the nest, and the lucky individuals were placed to the nursery aquaria. The egg mortality data of these offspring could not be recovered either. After these occasions the incubation times were shortened by one day in all groups. As a result the last days of the parenting period include fewer replicates so the data show less accuracy. However, these data are also shown in the graphs as an illustration (Figure 10 and 11).

5.1 Males

Group 19°C

Males in elevated temperature reached their optimal condition much faster. They were less scared and bolder. Most of them reacted less to disturbance. Their handling was easier, every aspect went smoother and they showed more interest in the females, which also meant they were inpatient and aggressive which sometimes even prevented the spawning because the female stressed out.

Group 14°C

The colour of the males was pale grey and brown instead of red, often mimicked the shade of the sand, their interest of the females was lower. Most of them were shy and scared, and their aggression level towards the females was more moderate and less frequent. Their agility of caring the offspring was low, but was not blocked. Each managed to spawn and most of them kept their eggs alive until hatching.

Table 1. Changes in the body weight (mean ± S.D.) of the males divided into groups and cycles.

Group/cycles	Start weight (g.) ± S.D.	End weight (g) ± S.D.	Weight loss (g) ± S.D.	Weight loss (%) ± S.D.
19°C 1st	2.59 ± 0.59	1.91 ± 0.49	0.68 ± 0.22	26 ± 7
19°C 2nd	2.39 ± 0.49	1.75 ± 0.41	0.63 ± 0.27	26 ± 9
14°C 1st	2.34 ± 0.42	1.94 ± 0.49	0.40 ± 0.24	17 ± 11
14°C 2nd	2.58 ± 0.55	2.13 ± 0.49	0.45 ± 0.24	17 ± 9

The data (Table 1) show that individuals in warmer temperatures lost more weight than in 14°C. Although the difference does not seem very large, the size of the animal should also be taken into account. The weight loss difference between 14°C and 19°C in the first cycle was significant (pairwise *t* test, t= -3.425, p=0.004), but less strong (pairwise *t* test, t= -1.534, p=0.147) in the second cycle. When comparing the 19°C group first cycle with the 14°C group second cycle results (pairwise *t* test, t=-2.165, p=0.048) and in the case of 14°C group first cycle in comparison with 19°C group second cycle the (pairwise *t* test, t=2.950) p=0.011. The difference is significant in 3 out of 4 cases and the 19°C had higher weight loss in all comparison.

5.2 Parenting behaviour, fanning of the nest

The males in the group 14°C had altogether 217 incubation days, 7-8 days male^{-1}; 120 days was without fanning the nest and 8 males have shown none or only little parenting care. The 19°C room had less, 5-6 days male^{-1} incubation time, altogether 120 parenting days in total; 35 absent days from parenting care and 1 male had low fanning time.

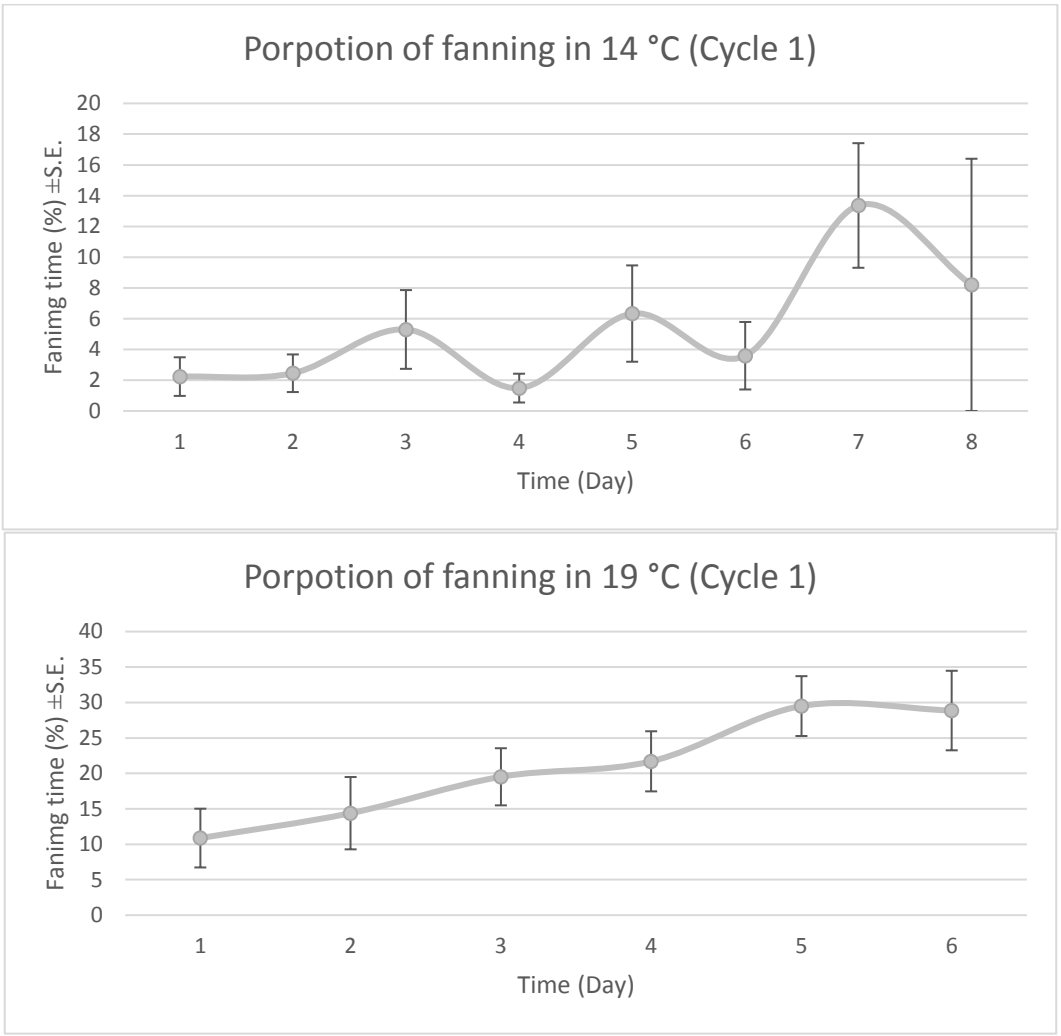

Figure 10. The average percentage ± standard error (S.E.) of the time males spent fanning the nest (Cycle 1, 14°C and 19°C).

As presented (Figure 10), the average fanning durations changed over time in both temperatures. The fanning behaviour began with lower values and increased, the highest peak was at the sixth and seventh day and decreased during the eighth day (n=5) in the 14°C group. The 19°C group did similarly, started with shorter fanning times and then increased and the most intensive fanning occurred during the last days (fifth and sixth) (n=11). When observing the cycle graphs; the males in 19°C spent between 10 and 30% time oxygenating the eggs, whereas the percentage was much lower at 14°C, between 2-13% with larger daily fluctuations than at the warmer temperature.

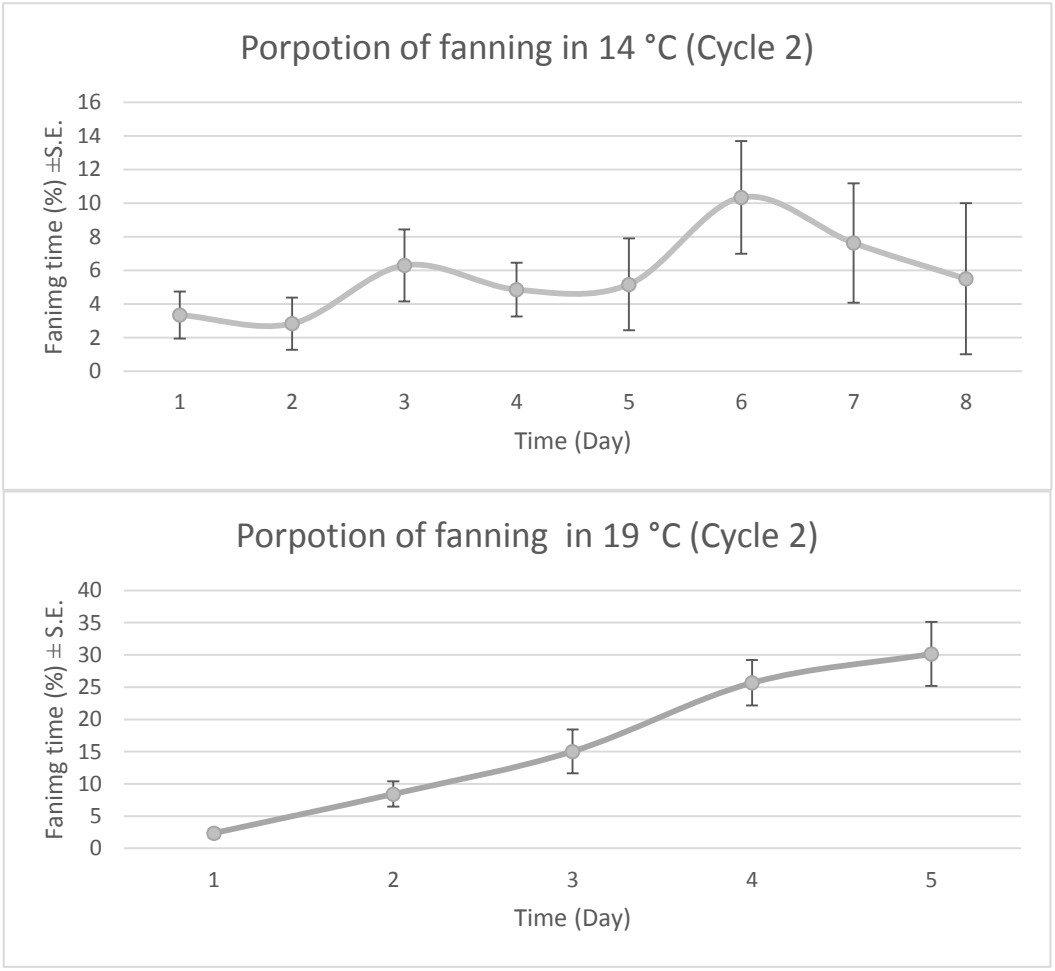

Figure 11. The average percentage ± standard error (S.E.) of time males spent fanning the nest (Cycle 2, 14°C and 19°C).

In the second cycle we can see similar patterns as (Figure 11) in the first cycle which means a slow start and a constant rise until the end. The highest peak is at the sixth day and decrease from here to the eighth day (n=3). The 14°C group has spent even less time fanning this cycle, only between 3 and 10% of the time was used to supply the offspring with oxygen while the group at 19°C shows more similar results to the first cycle. The 19°C group shows a bit larger changes this time between its lower and upper values from 3 to 30%. When comparing with the 14°C group, the rise seems to be much more restrained and more balanced throughout the process with less fluctuation. The 14°C and 19°C group gives similar results in both cycles even though 50% of the males were originally in the other group.

Table 2. Friedman's one way-ANOVA test from the fanning duration data, divided into cycles and subgroups with males unchanged and changed, p<0.005 is significant).

Ranks		**Test Statistics**	
	Mean Rank	N	39
14°C Cycle 1	2,53	Chi-Square	41.467
14°C Cycle 2, Original	2,53	df	5
14°C Cycle 2, Changed	3,41	Asymp. Sig	.000
19°C Cycle 1	3,94	a. Friedman test	
19°C Cycle 2, Original	4,18		
19°C Cycle 2, Changed	4,42		

To see if the observed differences between the two groups and cycles were significant, Friedman's ANOVA test was run on the fanning (sec.) data. The Friedman's test shows that the group means (14°C and 19°C Cycle 1, 14°C and 19°C Cycle 2 with original males, 14°C and 19°C Cycle 2 with the exchanged males) are not the same, except for 14°C cycle 1 and Cycle 2 Original males (Table 2, see more details in appendix). Differences are observable between the Original males and the Changed males which shows that the males coming from the other temperature group do not behave entirely in the same way as the original group. The differences are significant between the groups and subgroups in general ($p<0.001$) which would reject the $H0_1$ that there are no differences (the distribution of these variables are equal) in relation to the temperature. To be more accurate, and to see where the differences exactly were, a post-hoc Wilcoxon test was run.

Table 3. Wilcoxon Signed Ranks Test statistics results on fanning data, (p<0.05).

Compared against	p values
14°C Cycle 1 – 19°C Cycle 1	0.000
14°C Cycle 1 – 14°C Cycle 2 Original	0.933
14°C Cycle 1 – 14°C Cycle 2 Changed	0.001
14°C Cycle 2 Original – 14°C Cycle 2 Changed	0.001
19°C Cycle 1 – 19°C Cycle 2 Original	0.394
19°C Cycle 1 – 19°C Cycle 2 Changed	0.547
19°C Cycle 2 Original – 19°C Cycle 2 Changed	0.971
14°C Cycle 2 Original – 19°C Cycle 2 Original	0.000
14°C Cycle 2 Original – 19°C Cycle 2 Changed	0.000
14°C Cycle 2 Changed – 19°C Cycle 2 Original	0.001
14°C Cycle 2 Changed – 19°C Cycle 2 Changed	0.003

The cycles, groups and subgroups were tested against each other. The results shows that the differences between the 14°C and 19°C groups in the first and also in the second cycle were significant ($p<0.001$ – $p<0.003$) (Table 3, see more details in appendix). The difference was not significant when comparing 14°C Cycle 1 with 14°C Cycle 2 Original males ($p=0.933$), 19°C Cycle 1 with 19°C Cycle 2 Original males ($p=0.394$), 19°C Cycle 1 with 19°C Cycle 2 Changed males ($p=0.547$) and 19°C Cycle 2 Original males with 19°C Cycle 2 Changed males ($p=0.971$). Which means that between the first and second cycle there was only one significant difference when comparing 14°C Cycle 2 Original and 14°C Cycle 2 Changed groups.

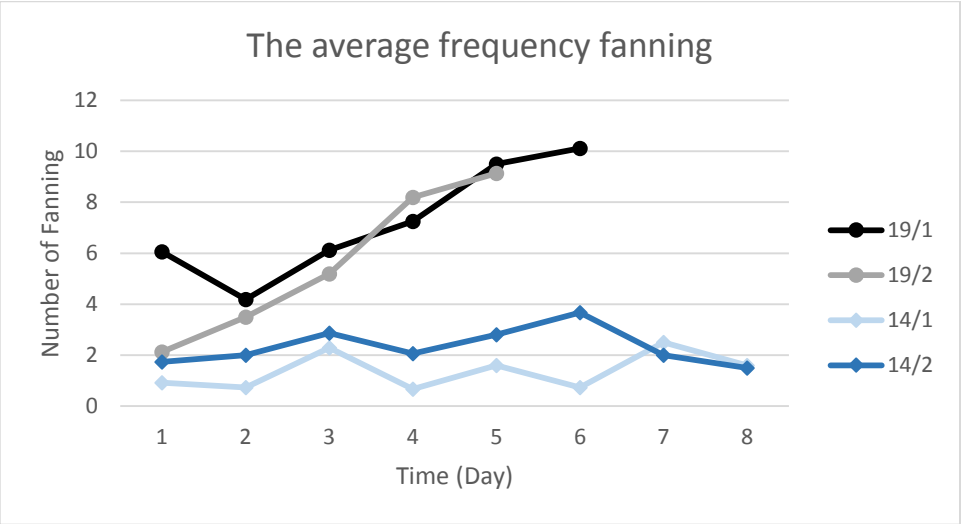

Figure 12. The average number of occasions for all groups and cycles when the males visited the nest for fanning.

It is also interesting to mention how many times the fanning volume is composed of. The number of occasions the males visited the nest shows how diligently and frequently the nursed their offspring. The frequency of fanning occasions (Figure 12) follows the similar form as the percentages of fanning, starting at a lower number and increases day by day. The males in 19°C in both cycles visited the nest more often than the other group. At 14°C the rise is more restrained and decreases a bit on the eighth day. There seems to be no large differences between the cycles.

5.3 No parental care

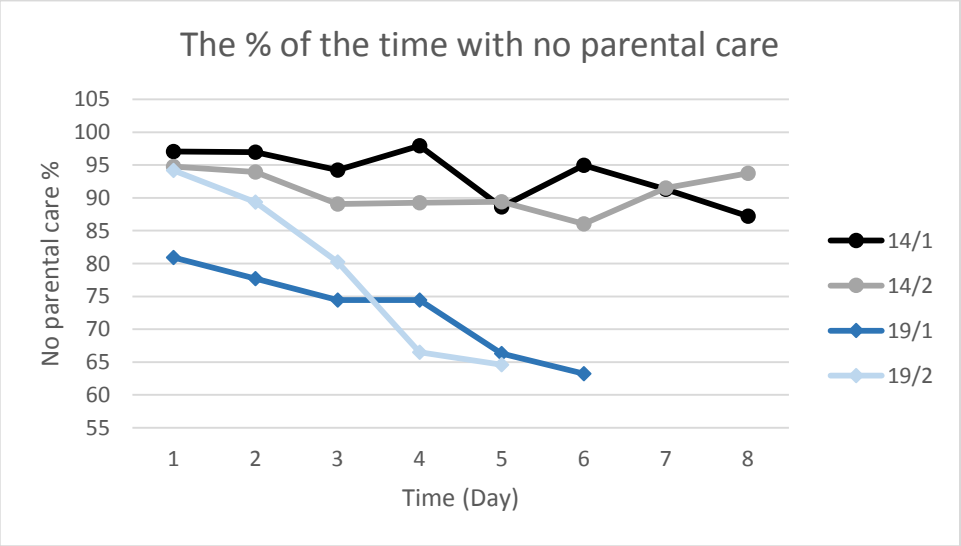

Figure 13. The percentage of time that the males in all groups and cycles has spent not caring for the offspring.

The number of times when males did not show any parental behaviour and did not visit the nest, i.e., there is no parental care marked as “no parental care” (Figure 13). As expected, this shows more or less similar but opposite extent patterns as the fanning graphs (Figure 10-11), meaning that the males in the 14°C group has spent more time not showing parental care while the 19°C group performed better.

5.4 Nose in the nest

Males occasionally push their noses into the nest during the fanning and for nest maintenance. This element of the parental behaviour is called “nose in the nest”. They also do this to poke holes into the nest to increase the water flow around the eggs. The result of the males’ performance in nose in the nest behaviour shows that the previously observed pattern (fanning the nest, Figure 10, 11), frequency of fanning (Figure 12), no parental care (Figure 13)) is not followed (Figure 14). Instead of the separation of the 14°C and 19°C groups shows that the structure of this behaviour seems to be random. The lowest value is in the 14°C cycle 1. The second cycle poked its nose to the nest more frequently than the in the second cycle.

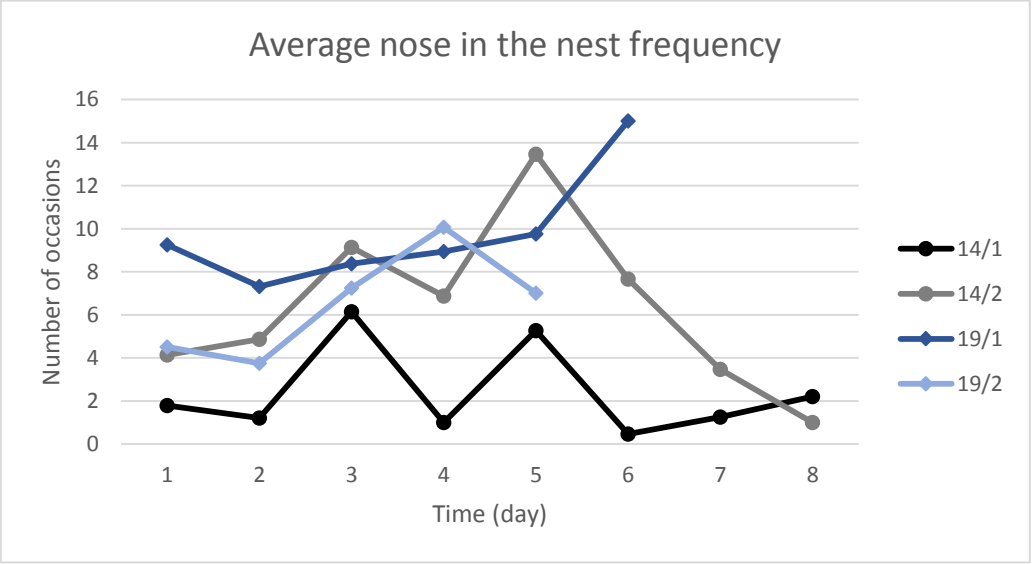

Figure 14. The average number of nose in the nest behaviour.

5.5 Eggs

The average diameter of randomly chosen eggs was 1860.83 ± 634.30 μm (⌀ mean ± S.D., n=360). The actual number of the eggs was not counted since it is not important in the current study. The mass of the egg cluster was highly variable; which may be influenced by the size of the female and consequently, the amount of eggs in them, plus the quantity of water absorbed by the eggs in 2 hours. During the experiment, not all males succeeded in keeping the offspring alive. Most of the males lost some of their eggs, which is an ordinary occurrence.

Table 4. The mean weight (± S.D.) of eggs at time of laying and before hatching, and before divided into groups and cycles, including survival.

Group	Weight day 1 (g) ± S.D.	Weight last day (g) ± S.D.	0 surviving	Weight loss (%) ± S.D.
19°C 1st	0.56 ± 0.21	0.44 ± 0.26	1	28 ± 35
19°C 2nd	0.54 ± 0.20	0.42 ± 0.25	2	26 ± 33
14°C 1st	0.65 ± 0.28	0.44 ± 0.31	3	34 ± 44
14°C 2nd	0.58 ± 0.28	0.43 ± 0.27	2	31 ± 36

The weight of the eggs clutch had high variance and its value changed over time in all groups (Table 4). However, no large differences between 14°C and 19°C groups (pairwise *t*-test, t=0.732 p=0.476, n=15 for cycle 1, and t=0.492 p= 0.630, n=15 for cycle 2) can be observed the different temperature groups, the 19°C groups performed better to keep their offspring alive than the 14°C ones. The deviations were usually negative, but in some cases the egg weight was heavier (n=7) at the end which might influence the weight loss results by lowering the average difference and acquiring a larger Standard Deviation (S.D.). Both groups contained individuals, which did not manage to keep a single egg alive; the value was slightly higher in 14°C.

5.6 Fries

The fry that succeeded to hatch and survive at least one week, were measured. After the first measurement, twenty (if there was that many) offspring per male were monitored separately over the second week. The survival rates of one-week-old juveniles varied widely. The 14°C group first cycle fry (fry group n=11) death rate was 3.54 ± 4.16 (mean ± S.D.) individual, which is 18% mortality, while in the 19°C group it was 15.88 ± 4.16 (mean ± S.D.), which is 75%. For the second cycle there was no fry data for the 14°C group, subsequently their hatching time ran over the study time. Some data was obtained from the group 19, where the fry mortality was 17.29 ± 3.99 (mean ± S.D., fry group n=6), 86%, which is higher than in the first cycle. The mortality difference between the temperatures is quite remarkable. The different temperatures in the environment indicate statistically significant changes in the fry mortality by giving $p<0.001$ value (two sample *t* test, t=7.94, n=13 and 16, Cycle 1 only).

Table 5. The average size ± S.D. of the fry in all groups and cycles after the first and second week.

Group	1 week size (µm) ± S.D.	2 week size (µm) ± S.D.
19°C 1st	6741.48 ± 872.74 (n=80)	7109.02 ± 1063.90 (n=59)
19°C 2nd	6644.25 ± 1050.5 (n=45)	6784.74 ± 262.58 (n=12)
14°C 1st	7013.33 ± 464.37 (n=55)	7203.83 ± 1193.75 (n=41)
14°C 2nd	6402.82 ± 1155.75 (n=35)	NA

The fry size data (Table 5) were incomplete due to the mortality and lack of time. Nevertheless there were measurements available with smaller sample size except for the second week of the 14°C group.

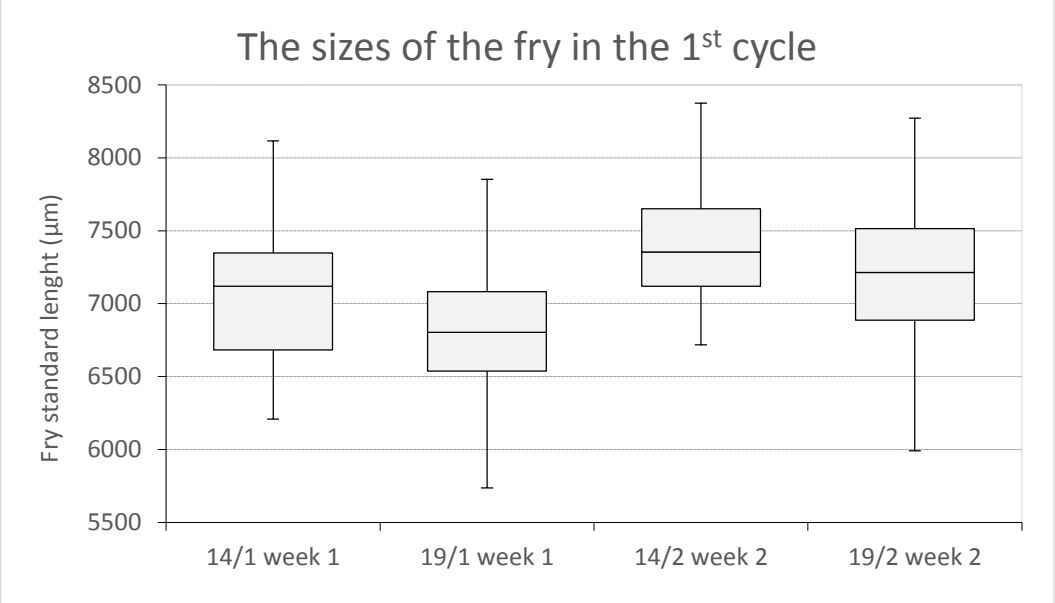

Figure 15. ***The size of the fry after 2 weeks in both temperatures (median, interquartile range (height of the box), max and min).***

When comparing the two temperatures in the first cycle for the period of two weeks of growth (Figure 15), an unexpected phenomenon can be observed showing that growth rates of fry at colder temperatures were slightly faster, and keeping this tendency over the second week in comparison with those growing at higher temperatures. The difference between the 14°C and 19°C groups were significant (two sample *t* test, t=2.40, n=80 in 19°C and 55 in 14°C, p=0.018) in the first week but less remarkable in the second week (two sample *t* test, same t=1.52, n=59 in 19°C and n= 41 in 14°C, p=0.132). The comparison is made only from the 1st cycle because the data were not satisfactory from the second cycle.

6 Discussion

My results showing that temperature changes the three-spined stickleback's reproduction, parental care and fry fitness are supported by the original observation made by Van Iersel (1953) too. I also noticed high variability between individuals, regardless of the temperature, as shown by Stein & Bell (2012). The gap between the two different temperatures seems wide even if the temperature difference is 5°C. Some males were agile and started to build their nest and the court behaviour directly, and some were a bit careful and needed some encouragement and peace. Both groups contained really shy and really aggressive or opportunistic individuals, but perhaps the level of aggression was higher at 19°C. Nevertheless, it can be stated that significant alterations in the males' behaviour can be observed due to the temperature differences. Elevated aggression and bright colouration was

common for males in 19°C, which in some cases disturbed the spawning, because the male's aggressive behaviour scared away the female preventing the spawning. On the other hand, the eggs were often laid faster here regardless of the aggressive behaviour. The males at 19°C also showed very intense parenting care. The eggs hatched approximately 3 days faster than in the 14°C group. Even though the greater parenting effort, the egg survival rate was not significantly different. During the same time the males in 19°C had lost more weight. Loosing approximately the ¼ of their body weight could affect their condition and odds of survival and performance during the next breeding cycle. Despite, the males' mortality was higher at 14°C than at 19°C.

Four elements of the parenting behaviour observation is the *total time of the fanning*, the *number of times when visiting the nest for fanning*, the time when there is *no parental care* and the time when the *male's "nose" was in the nest*. Maybe the energy consuming fanning behaviour is the most significant (Van Iersel 1953, 23). Of course, this is a very simplistic view of the parental behaviour of a species, which is much more complex and includes many more features, but these were the easiest to measure (Van Iersel 1953, 25). In order to verify the accuracy of the illustrations presented above, it was necessary to include Standard Error(s) (S.E).

6.1 Parental success

Parental success is a fundamental aspect of the breeding period in terms of population growth. The male mortality is less vital since, they would possibly die at the end of the season anyway. As stated by Stein & Bell (2012) there is no evidence that the size of the egg clutch would influence the parenting performance of the males so hypothetically all differences observed should be originated from the environmental circumstances or the males' individual properties. Because of the mortality of the males and the fact that they needed to be replaced with new males and start the cycle all over, there was no time to wait for all the offspring to hatch or reach the age of 2 weeks, so the information on the development of the offspring is incomplete.

6.1.1 Fanning

In the warmer temperature, males spent additional time ventilating the offspring in both cycles regardless if the male was in the warmer room from the beginning or was transferred there at the end of the first cycle. The embryos need oxygen which is less abundant in warmer water, which motivates males to fan more to compensate the lower oxygen level. This suggests that these differences were actually indicated by the difference in temperature and oxygen level and not by the individual hereditary behavioural differences in males. According to Stein and Bell (2012), there is no correlation between the males' size and parental behaviour. It seems that males in unusually warm environment needed to fan more to keep their offspring alive than in normal condition.

The fanning intensity was rising from the first day till the end in both groups and cycles. Figures 10 and 11, the fanning patterns show that at 14°C the males fanned more casually. It could be presumed that they fanned when they wanted to or when their inner stimuli motivated them to rather than because of the eggs oxygen need. Conversely in 19°C, the fanning is more uniform without major fluctuations and with smaller standard deviations, which suggest they did the fanning because they needed to in order to keep their eggs alive. The last days of fanning show higher variation, possibly as a result of the low replication. Even so, the pattern of visiting the nest is still evident. The phenomenon was expected as males spend gradually more time caring for juveniles as hatching time approaches (Van Iersel 1953, 32). Van Iersel (1953) states also that this behaviour was based more on inner stimuli than the needs of the eggs. A few times the egg weight was higher at the time of removal, which could be a measurement error or due to the egg's mortality being low and absorbed water during development.

As a result there was no significant difference between males in the same temperature but in different cycles; only when comparing 14°C group cycle 2 original males and 14°C group cycle 2 changed males. The males being transferred from the 19°C group to the 14°C show different behaviour, but the ones being moved from the 14°C to the 19 adapted to the new circumstances and did not differ from the original males. This seems to support Van Iersel' (1953, 5) report that the males keep their changed behaviour after the first warmer period. If this is so, then the more frequent heat waves can influence the reproduction habits of the stickleback by influencing the males' behaviour. However, the 14°C group' first and second cycle did not vary from each other greatly after all.

6.1.2 Fries

One interesting information should be stated about the fry. Their survival rate was much higher in the colder temperature than in the warmer. Many times we managed to raise 20 out of 20 fry at 14°C, while this did not occur a single time in 19°C. The growth of fry seems to be faster in 14°C, which is different from what Glippa et al. (2017) observed. They found that the optimal temperature for the fry is 21.7°C, even higher than the elevated temperature in our study. Maybe if we could observe the growth rate for a longer time, it could give a different result since the size gap seemed to decrease after the first week.

6.2 Factors affecting the results

Keeping the temperature stable was challenging. Since everything was prepared manually it took days to find the optimal speed of the water flow. Although the temperatures and the water flow were continuously monitored, minor temporary oscillations occurred, possibly caused by the malfunction of the water pumping system in the building. This is however unlikely to influence the results to any greater extent.

The males in the 14°C room had much weaker motivation to build the nest and to protect the offspring. A common problem was that the female did not fit, or barely fitted into the nest, and when she forced herself inside, the nest was destroyed. When this happened the male tried to fix it quickly, but the female was often dissatisfied and refused the spawn. A few females needed to be replaced because of this. Unfortunately, the eggs hardened inside the female's body in some cases, and these eggs could not be laid. These females are no longer able to spawn in their life and likely to die as a result. The other extreme was when the females laid the eggs in the containers during the waiting time between the courtship recordings (that data are examined by another student). In both cases, a new female is needed. Another unexpected problem was also the larvae untimely hatching; despite the fact that Van Iersel (1953, 78) measured 6.6 days to hatch at 18.8°C, our larvae at 19°C repeatedly hatched during the fifth or even fourth day. The same happened in the 14°C group; instead of the expected 8 days they hatched at the seventh, but not as frequently as in the other group. After the breeding cycles, all female were released back to the sea near the station.

6.3 Limitation of the study and future suggestions

The influence of stress during the experiment cannot be entirely excluded because the fish were kept in unnatural conditions, and are sensitive to noise and strong light. The rooms were used by other researchers at the same time, and they might have disturbed the males.

The effects of warmed water on early survival of juveniles should be studied more. Undeniably with such a small number of replicates, a reliable result may not necessarily be obtained, but hopefully the research will continue in the coming years by other students. The reason for the high mortality of the offspring at 19°C should be further investigated. It might not be a direct consequence of the temperature, maybe it occurred because the different oxygen level of the tanks, or as an effect of the "bad water" as Van Iersel (1953, 39) stated. Other reasons could be increased decay of excess food or even the different state of bacterial and algal community in the tanks, or just a technical error. In this instance, it would also be an indirect result of the temperature and may also take place in the sea.

7 Conclusion

Due to the climate change the warmer periods when the temperature elevate above normal is becoming more frequent. When the temperature rises, the environment and the breeding habits of stickleback change. Stickleback males can get into reproductive stage faster so their breeding season can start earlier. Their breeding attitude can increase and so does the aggression of the males. Although the hatching success of the offspring was slightly but not significantly higher, their chances of survival later worsened.

The outcomes of the research, unquestionably, do not necessarily reflect all the processes taking place in the Baltic Sea, since the fish were in a non-natural environment, which does not correspond to all parameters observed in the wild. Nonetheless, it can be assumed that the change in temperature may have a similar effect on the males as we experienced in the laboratory. It should also be mentioned that in order to achieve reliable results the sample size should be increased. The long-term consequences are difficult to predict. According to the research, warming is not necessarily beneficial for the stickleback, but in the Baltic Sea the population size is constantly increasing, suggesting that other processes may also contribute.

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Appendix

Friedman's ANOVA Descriptive Statistics details on fanning duration data

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Cycle1Cold	39	15,27	31,073	0	121
Cycle2Original1	39	21,21	48,060	0	244
Cycle2Changed1	39	37,57	43,350	0	152
Cycle1Warm	39	89,1256	113,22273	,00	418,93
Cycle2Original	39	102,0969	110,73953	,00	387,78
Cycle2Changed	39	98,6674	96,20597	,00	276,28

Wilcoxon signed ranks test result details on fanning duration data

Ranks

		N	Mean Rank	Sum of Ranks
Cycle1Warm - Cycle1Cold	Negative Ranks	14[a]	24,21	339,00
	Positive Ranks	67[b]	44,51	2982,00
	Ties	9[c]		
	Total	90		
Cycle2Original1 - Cycle1Cold	Negative Ranks	23[d]	29,22	672,00
	Positive Ranks	28[e]	23,36	654,00
	Ties	40[f]		
	Total	91		
Cycle2Changed1 - Cycle1Cold	Negative Ranks	12[g]	23,00	276,00
	Positive Ranks	36[h]	25,00	900,00
	Ties	9[i]		
	Total	57		
Cycle2Changed1 - Cycle2Original1	Negative Ranks	8[j]	26,31	210,50
	Positive Ranks	36[k]	21,65	779,50
	Ties	13[l]		
	Total	57		
Cycle2Original - Cycle1Warm	Negative Ranks	17[m]	17,35	295,00
	Positive Ranks	20[n]	20,40	408,00
	Ties	3[o]		
	Total	40		
Cycle2Changed - Cycle1Warm	Negative Ranks	15[p]	21,93	329,00
	Positive Ranks	23[q]	17,91	412,00
	Ties	1[r]		
	Total	39		
Cycle2Changed - Cycle2Original	Negative Ranks	18[s]	20,44	368,00
	Positive Ranks	20[t]	18,65	373,00
	Ties	1[u]		
	Total	39		
Cycle2Original - Cycle2Original1	Negative Ranks	7[v]	9,29	65,00
	Positive Ranks	27[w]	19,63	530,00
	Ties	6[x]		
	Total	40		
Cycle2Changed - Cycle2Original1	Negative Ranks	5[y]	10,80	54,00
	Positive Ranks	30[z]	19,20	576,00
	Ties	4[aa]		
	Total	39		
Cycle2Original - Cycle2Changed1	Negative Ranks	12[ab]	11,75	141,00
	Positive Ranks	26[ac]	23,08	600,00
	Ties	2[ad]		
	Total	40		
Cycle2Changed - Cycle2Changed1	Negative Ranks	12[ae]	14,75	177,00
	Positive Ranks	27[af]	22,33	603,00
	Ties	0[ag]		
	Total	39		

a. Cycle1Warm < Cycle1Cold

b. Cycle1Warm > Cycle1Cold

c. Cycle1Warm = Cycle1Cold

d. Cycle2Original1 < Cycle1Cold

e. Cycle2Original1 > Cycle1Cold

f. Cycle2Original1 = Cycle1Cold

g. Cycle2Changed1 < Cycle1Cold

h. Cycle2Changed1 > Cycle1Cold

i. Cycle2Changed1 = Cycle1Cold

j. Cycle2Changed1 < Cycle2Original1

k. Cycle2Changed1 > Cycle2Original1

l. Cycle2Changed1 = Cycle2Original1

m. Cycle2Original < Cycle1Warm

n. Cycle2Original > Cycle1Warm

o. Cycle2Original = Cycle1Warm

p. Cycle2Changed < Cycle1Warm

q. Cycle2Changed > Cycle1Warm

r. Cycle2Changed = Cycle1Warm

s. Cycle2Changed < Cycle2Original

t. Cycle2Changed > Cycle2Original

u. Cycle2Changed = Cycle2Original

v. Cycle2Original < Cycle2Original1

w. Cycle2Original > Cycle2Original1

x. Cycle2Original = Cycle2Original1

y. Cycle2Changed < Cycle2Original1

z. Cycle2Changed > Cycle2Original1

aa. Cycle2Changed = Cycle2Original1

ab. Cycle2Original < Cycle2Changed1

ac. Cycle2Original > Cycle2Changed1

ad. Cycle2Original = Cycle2Changed1

ae. Cycle2Changed < Cycle2Changed1

af. Cycle2Changed > Cycle2Changed1

ag. Cycle2Changed = Cycle2Changed1